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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

A histological evaluation of bone tissue response to a sealer based on calcium hydroxide – An experimental study

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SUMMARY
Introduction/Objective The success of endodontic treatment depends on the effective removal of root content, the elimination of infection, and the hermetical sealing of the root system using a compatible material.
The objective of this paper was to evaluate the tissue response to the implant of endodontic material based on calcium hydroxide into the bone in the artificially prepared defect in a rat mandible.
Methods The research was carried out on 40 Wistar rats. The artificial defect was made between the midline and the mental foramen on the left side of the mandible. The prepared defect was left to heal spontaneously in animals of the control group, while among the animals of the experimental group the sealer Apexit (Ivoclar Vivadent, Schaan, Lichtenstein) was implanted into the experimental defect. The tissue samples consisting of the experimental field and the surrounding bone were microscopically analyzed with a light microscope.
Results During the initial phase, 15 days after the implantation, signs of chronic inflammation were noted and expansion of the Volkmann's and Haversian canals. On the 30th day after the implantation, osteosynthetic activity and filling with newly-formed bone were noted. Changes were also noted in cement lines in the wider region of the experimental defect. Sixty days following the implantation, the bone was gradually remodeled. Ninety days after the implantation, a *restitutio ad integrum* was noted.
Conclusion Apexit does not lead to any disruptions in normal reparation processes nor in morpho-functional relations in bone tissue during the remodeling phase.
Keywords: endodontic sealers; calcium hydroxide; bone; healing

INTRODUCTION

The success of endodontic treatment depends on the effective removal of root content, the elimination of infection and the hermetical sealing of the root system using a compatible material. This concept cannot become outdated, and is constantly being improved through new techniques, methods, and the use of new materials and instruments [1].

The material used for teeth sealing remains permanently in the root system, so its influence on the surrounding tissue is possible. This material, during the hardening process, can release certain components (especially in the unbound state) which can damage the surrounding structures [2].

The frequent failure related to endodontic therapy is more significant in the case of overfilled root canals than in the case of canals which were sealed to a precisely determined working length. Histological analyses of periapical tissue following overfilling indicate increased inflammation and delayed healing [3, 4]. Certain authors believe that the material which is minimally transferred over the apex can be a healing stimulus [5].

On the other hand, the material can influence the outcome of the healing process, as a result of its chemical characteristics (electrochemical reactions, the content of strong disinfectant and cytotoxic components) [6], physical features (solubility, density, etc.) [3], or its biological features (biocompatibility, healing stimulation, etc.) [7].

Sealers based on calcium hydroxide are used to permanently seal the root canal of a tooth, among other things, as a result of possible influence on the healing of the damaged periapical tissue and antimicrobial effect [6, 8, 9].

The influence on healing takes place due to the following: an alkaline pH value [9], which maintains the bone metabolism in a constantly mild alkaline environment [10], which in turn contributes to the neutralization of the acid metabolites of macrophages and osteoclasts [11], and stimulates the activity of the calcification enzymes of the osteoblasts [12].

In addition to the high pH value, the beneficial effect of the sealer based on calcium hydroxide on the process of hard tissue regen-

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eration is realized in part due to the presence of calcium and phosphate ions [9, 13, 14], the capacity to attract blast cells [15], the promotion of angiogenesis with the growth factor concentration [10], the expression of the human bone sialoprotein gene [16], the creation of a favorable environment for the formation of cement [15, 17], and the bone conduction and cement conduction effect [18].

The antimicrobial effects of a sealer based on calcium hydroxide are manifested by its influence on the growth, metabolism, and division of cells, which indirectly favors the synthesis of mineralized structures by the reduction of inflammation [10], as well as the dissolution of the rest of the necrotic tissue [15, 13].

In order to reduce the systematic and local harmful effects of the material to a minimum, it is necessary to use an obturating material which does not irritate the tissue it is in contact with via the apex opening and access canals.

The objective of this paper was to evaluate the tissue response to the implant of endodontic material based on calcium hydroxide into the artificially prepared defect in a rat mandible.

METHODS

The research was carried out on a sample of 40 Wistar rats, all males, aged six to eight weeks, average body mass of 160–180 grams, with the approval of the Ethics Committee of the Faculty of Medicine in Niš (No. 01 3797). The animals were prepared for the intervention by anesthesia using ketamine hydrochloride (Ketamidor 10%, Richter Pharma AG, Wels, Austria) in doses of 0.1 ml / 100 g of body mass. Following the anesthesia, the artificial defect was made between the midline and the mental foramen on the left side of the mandible (1.4 mm in width, 1.6 mm in depth) by using sterile steel burs.

The prepared defect was left to spontaneously heal in 16 animals of the control group, while among the animals of the experimental group (24 rats) the sealer Apexit (Ivoclar Vivadent, Schaan, Lichtenstein) was implanted into the defect, whose content is shown in Table 1.

The animals were sacrificed with an overdose of anesthetic 15, 30, 60, and 90 days after the implantation. Accordingly, four subgroups were formed, and each numbered four animals from the control group, and six animals from the experimental group.

Extraction of tissue samples was carried out through the resection of the mandible and consisted of the experimental field and the surrounding bone. The samples were fixed in 10% buffered formalin, demineralized in 10% formic acid, dehydrated in a series of graded alcohols, and cleared with benzene and embedded in paraffin wax. The cutting was performed in a buccolingual direction using a microtome (Historange) with glass knives 2 μm wide. The material was stained using hematoxylin-eosin and microscopically analyzed with a light microscope BX50 (Olympus Corporation, Tokyo, Japan) fitted with a DFC 295 digital camera (Leica Camera AG, Wetzlar, Germany).

Table 1. The content of Apexit

Base	Activator
Bisphenol A diglycidyl ether	Trimethyl hexanediol disalicylate
Calcium hydroxide	Bismuth carbonate
Rosin hydrogenated	Bismuth oxide
Silicon oxide	Silicon oxide
Calcium oxide	1,3-butanediol disalicylate
Zinc oxide	Rosin hydrogenated
Tricalcium phosphate	Tricalcium phosphate
Polydimethylsiloxane	Zinc stearate
Zinc stearate	
Paraffin oil	

RESULTS

The samples of the artificial defects of the mandible obtained from the control and experimental groups, which were microscopically studied in chronological order, showed relatively stable morphological findings of bone healing. The obtained results are shown in Figures 1–8.

During the initial phase, 15 days following the application of the freshly mixed material (in all six animals of the experimental group), early resorptive callus tissue was noted, i.e. fibro-vascular connective tissue with a moderate number of cells showing signs of chronic inflammation. Changes were noted in the endosseous system in the vicinity of the experimental defect, and included the expansion of the Volkmann's and Haversian canals with an increase in the volume of their connective content. The prominent basophilic osteoblasts were set in one layer towards the walls of the defect, while the presence of the osteoclast was inconspicuous and on the periphery of the callus (Figure 1). Figure 2 shows a control sample dating from the same elapsed time interval.

On the 30th day following the implantation, a decrease in the callus volume was noted and osteosynthetic activity with filling in by the newly formed bone (among five of the six animals from the experimental group). There was pronounced development of the endosseous communication of the Volkmann's and Haversian canal type. The

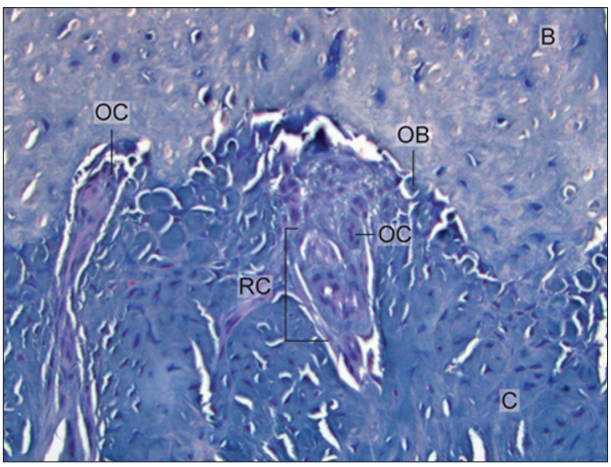

Figure 1. The experimental group – day 15; the periphery of the callus within the cavity of the experimental defect; the fibro-vascular structure of the callus (C) indicates a close physical relationship towards the environment of the bone (B) via the osteoclast (OC) on the 'enlarged' side of the vascular resorption cone (RC), as well as rare osteoblasts (OB) in the remaining borderline area; (HE, ×400)

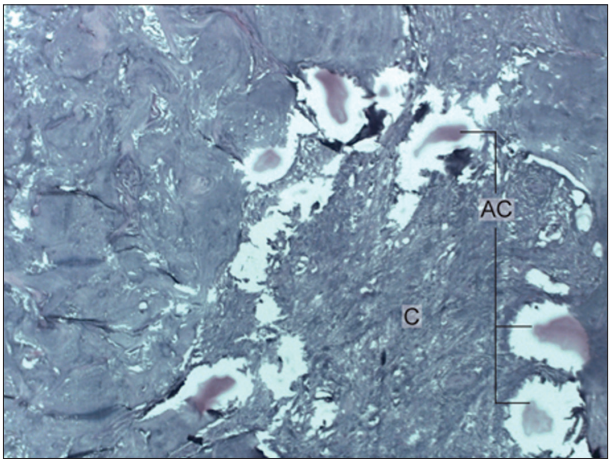

Figure 2. The control group – day 15; the callus (C) of the experimental defect with the peripheral artificial cavities (AC) which occurred as a result of the deformation of the extracellular matrix of the surrounding bone during the processing of the material; (HE, ×200)

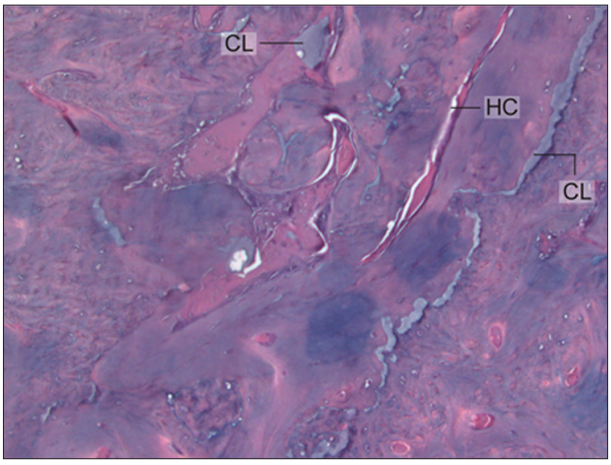

Figure 3. The experimental group – day 30; a combination of the lacunar changes in the cement lines (CL) and extensions of the endosseous holes (the Volkmann's and Haversian canals (HC)) in the bone tissue not in the vicinity of the defect border; (HE, ×200)

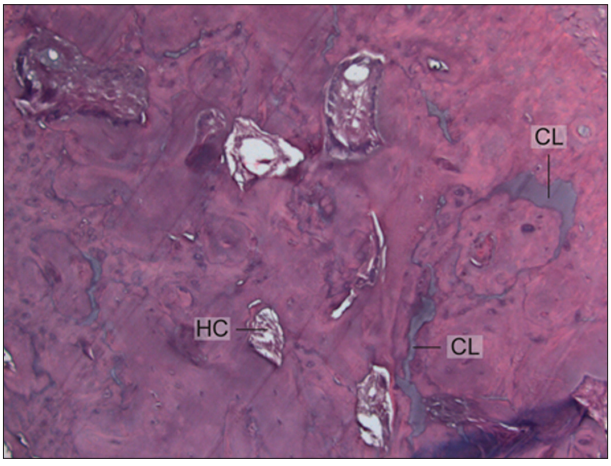

Figure 4. The control group – day 30; in the wider region in relation to the edge of the defect, especially in the osteon borders, the cement lines (CL) are prominent, to the edge of a fissure and irregular polygonal shape, filled with granulation and amorphous material; extended Haversian canals (HC); (HE, ×200)

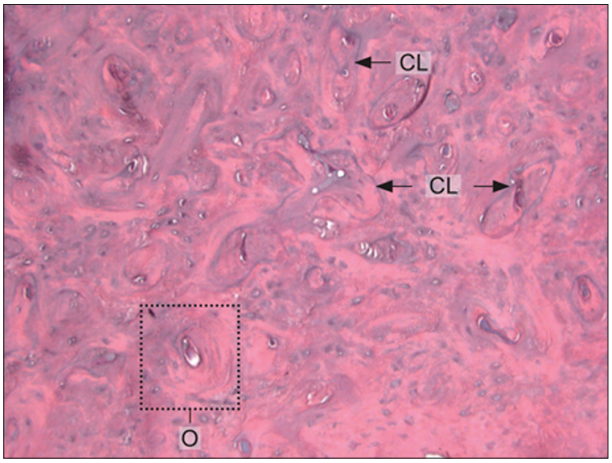

Figure 5. The experimental group – day 60; a greater number of larger osteons, mutually separated by a wide interstitial lamellae in which the osteocytes in the oval lacunae can be found; the cement lines (CL) are prominent on the edges of the osteon (O); (HE, ×200)

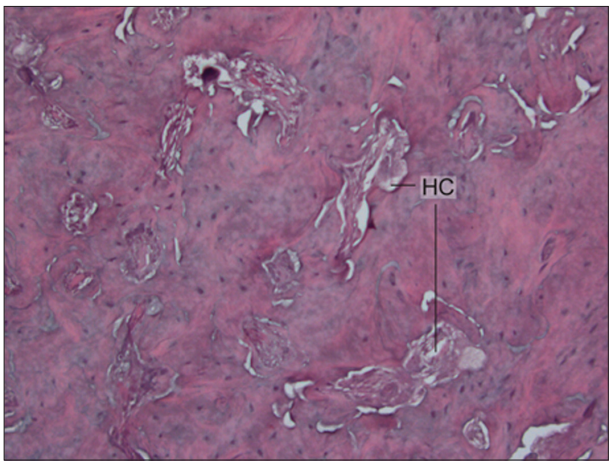

Figure 6. The control group – day 60; a newly formed bone with irregular mineralization with osteons, relatively wide Haversian canals (HC), and wide interstitial lamellae are noted; the bone is in gradual remodeling and in the process of maturation; (HE, ×200)

osteocytes were found in enlarged lacunae with a fortified basophilic border. Changes were also noted in cement lines in the wider region of the experimental defect in the form of a lacunar enlargement of the extracellular matrix between the osteon and interstitial lamellae. These regions showed variations in terms of size and shape, ranging from linear to wide periosteal lacunar formations, filled with granulation or amorphous material of a basophilic reaction (Figure 3). Figure 4 shows the control sample following the lapse of the same time interval.

Sixty days following the implantation (in five of the six animals in the experimental group), a lamellar bone was noted, with a relatively large Haversian system, with wide, undulating interstitial lamellae, as well as sporadic lacunar extensions of the extracellular matrix at the level of the cement lines. Even though uneven mineralization was noted, the bone was gradually remodeled and the vascular component was reduced (Figure 5). Figure 6 shows a control sample following the lapse of the same time interval.

Ninety days after the implantation, a *restitutio ad integrum* was noted along with the complete filling of the experimental cavity with bone tissue made up of numerous

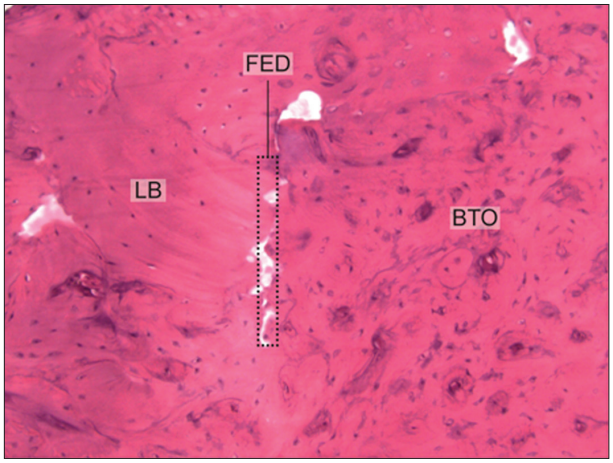

Figure 7. The experimental group – day 90; the sharp transition from the organization of bone tissue (BTO) based on the type of osteon towards the lamellar bone (LB) in the vicinity represents the edge of a former experimental defect (FED); (HE, ×200)

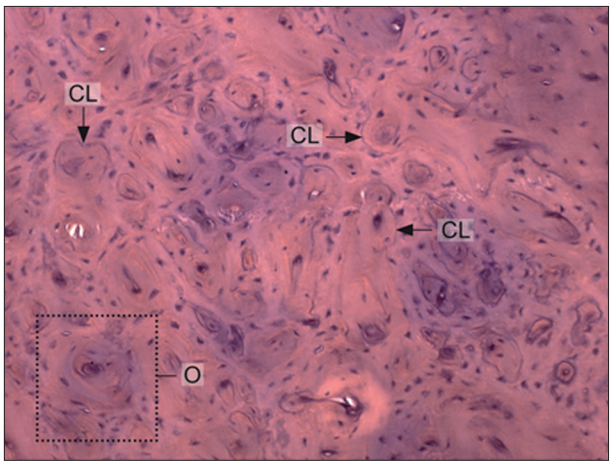

Figure 8. The control group – day 90; the complete healing of the bone tissue in the area of the former experimental defect consisting of numerous osteons (O) and a smaller number of concentric lamellae and cement lines (CL); (HE, ×200)

osteons of a smaller diameter, with a smaller number of concentric lamellae, whose outer border is characterized by a cement line with an increased basophilic reaction (in all six of the animals of the experimental group) (Figure 7). Figure 8 shows the control sample following the lapse of the same time interval.

In addition to the morphological features of healing noted in the location of the experimental damage (in the group of animals where the defect was filled with Apexit and also among the animals of the control group), a sequence of morphological changes was noted at a maximum distance of 3 mm from the edge of the defect, which depended on the chronological wholes of the experiment.

Within the experimental defects (in all the studied time intervals), no presence of the obturating material was noted.

DISCUSSION

In order to study the biocompatibility of the endodontic material, *in vitro* tests (on cell cultures) could be used along with *in vivo* tests (subcutaneous, intramuscular, and intraosseous implantation) [19]. The implantation techniques were more suitable since the healing processes cannot be simulated in the cell culture. On the other hand, intraosseous implantation cannot mimic the clinical situation of close contact of endodontic material with bone.

In this study, the method of intraosseous implantation into the mandible of a rat was used. This method requires great precision due to the requirement of the material to be built into the narrow space between the roots of the incisors and the first molars, which, in the case of these animals, are quite close to each other [20]. In order to avoid possible contact between the endodontic material and periodontal ligament, certain researchers recommend that implant testing should be carried out on the tibia and femur of small animals [21]. Irrespective of the aforementioned risk, it is considered that an implant into the mandible is more suitable, since the structural differences between the mandible and other bones influence the healing process, which is manifested in various reactions [20].

The aim of this experiment was to study the reaction of bone to an extreme experimental stimulant in relation to the homeostatic and normal morpho-functional elements. In such circumstances, there occurs a provocation of the normal pericavital structures which have to endure the influence of mechanical and thermal influence during the preparation, while the material that is additionally introduced into the defect becomes a physical and chemical obstacle in the bone defect reparation response.

In relation to the *restitutio ad integrum* which is expected to occur after day 35 in the case of rats [22], in this study the material did not lead to an extension in the reparation period, nor to the alteration in the bone tissue. The newly formed bone tissue showed signs of lamellar organization and completely filled in the defect, while no traces of sealer were noted in the defect. No asymmetry was registered in the progress of the reparation or a lack in the smaller cavities through which, when processing, the implanted material could be lost. The studied sealer cannot be considered the cause of the granulation and macrophage reaction, but only the normal state of inflammation. The noted minimal difference between the control and experimental groups lies in the frequent presence of basophilic cement lines on the periphery of the osteon in the group in which the obturation of the defect was carried out using Apexit. Both groups have shown an approximately similar tempo in achieving the morphological manifestations of reparation.

The obturation materials based on calcium hydroxide can initially cause an inflammation, which will over time decrease or completely disappear [2, 23]. A moderate to acute inflammation on the seventh day following the implantation was described by authors who implanted a sealer based on calcium hydroxide (Sealer 26) into the subcutaneous tissue of rats. The inflammation was recorded on day 42, but it was of a smaller intensity [2]. The inflammatory reaction on the seventh day following the implantation into the subcutaneous tissue of rats was also acute in the analysis of three different sealers based on calcium hydroxide (Sealapex, Apexit, Sealer 26). A sig-

nificantly milder inflammatory response was noted on day 21. The authors justify these results by the fact that the freshly mixed materials are more irritating and potentially cytotoxic [23].

Zmener et al. [4], in one of the two studied materials based on calcium hydroxide (CRCS), implanted into the subcutaneous tissue of rats, also described a decrease of the inflammatory response on days 30 and 90, but, also the increase in the inflammation in the case of the other studied material (Sealapex). The authors ascribe the obtained results to the content of the used material, such as titanium dioxide from the Sealapex, which is easily soluble in the tissue and can cause a foreign body reaction, and eugenol from the CRCS, which is a proven irritant.

Bernáth and Szabó [24] indicated the existence of a mild inflammatory response six months after the overfilling of root canals among primates using Apexit. In the experiment there was no inflammatory reaction in cases when the teeth were filled to the apex, and the authors explain their results by a lack of adherence to the biological concept and the specific nature of the reaction of the tissue of primates in relation to non-primates.

The results obtained in this study are not in accordance with the cited one since they indicate the presence of a chronic inflammatory response only 15 days after the implantation, which represents the natural course of the healing process. We can note a resorptive soft callus, but also a reaction in the bone tissue not in the proximity of the defect among animals sacrificed on the 15th and 30th day. Endosseous communication, of the Volkmann's and Haversian canal type, was significantly developed, which indicates that the bone tissue passes through a process of maturation and remodeling. Considering that the sealing material based on calcium hydroxide was the result of the addition of calcium hydroxide formulas to zinc oxide or resin [25], the amount and concentration of calcium hydroxide in them as well as the presence of other components, influences different tissue reactions to these materials [2]. In addition, the results are also influenced by the selection of animal models, tissue (subcutaneous, bone, filled or overfilled teeth), as well as the manner of implantation (injecting sealers into the tissue, introducing them using material such as silicon and Teflon).

Restitutio ad integrum following day 60 and day 90 with the maturation and remodeling of the bone is in accordance with the results found in the literature focusing on longer periods of implantation [24, 26, 4]. The authors explain this with the biocompatibility of the used material. The tissue gradually regenerates from the performed surgical trauma and the healing process ends.

The stimulation of calcification, only in cases when the studied material was based on calcium hydroxide, is confirmed by authors who implanted sealers into the subcutaneous tissue of guinea pigs [27]. The obtained results are ascribed to the presence of calcium hydroxide in sealers (CRCS, Sealapex) unlike other studied sealers (Endofill, Grossman sealer), which do not contain it. They emphasize the significance of their results since calcification was noted in the soft tissue. A similar attitude is shared by authors who determined that the rinsing of defects in the mandible of rats with a suspension of calcium hydroxide prior to the obturation of the mineral trioxide aggregate has a favorable effect. Collagen fibers are better organized and thicker, and the newly formed bone is trabecular, with wide blood vessels and a large number of osteocytes and osteoblasts. Analyzing the obtained results, the researchers cite that a suspension of calcium hydroxide stimulates the calcification enzymes of osteoblasts, and the high pH value provides an environment without bacteria [28].

By adding calcium hydroxide to portland cement, in an experiment focusing on the mandibular defects among dogs, did not lead to an increase in the regeneration process. The reason for that, according to the authors, could lie in the fact that the material with the addition of calcium hydroxide is more soluble, and that high concentrations of calcium hydroxide can be cytotoxic [10].

In a clinical study carried out on a sample of 204 teeth, the canals were filled with material based on zinc oxide (Proco Sol), and calcium hydroxide (Sealapex) or zinc oxide with the addition of calcium hydroxide (CRCS). A radiographic and clinical analysis after a period of two years showed the state of the periapex is best in the case of the Sealapex, while after three and four years there was no significant difference between the studied sealers. The difference in the studied period of two years the authors explain with different inflammatory responses to different material, as well as the fact that Sealapex has a greater alkaline value and calcium ion concentration in relation to the other two studied materials [29].

In this study, Apexit did not have a compromising effect on bone reparation, did not cause any contact inhibition, but there was also no significant stimulative healing effect. The stimulative healing effect of these sealers is expected of their component of calcium hydroxide. However, the manufacturer-provided information on the pH value of the material investigated in this study is 8.5, which is far lower than the pH which exceeds 12 in the case of pure calcium hydroxide, whose stimulating effect on mineralization has already been proven. On the other hand, Apexit is a biocompatible material, and does not contain significantly irritant components which could possibly impede the healing process.

Relying on the obtained results, we can assume that using this material as a sealer, even in cases of overfilling, does not diminish the biological potential of the bone to heal. This research has shown that the studied material did not lead to any permanent or significant disruption in the morpho-functional relations in the bone tissue following the studied intervals of 15, 30, 60, and 90 days.

CONCLUSION

Apexit, as a sealing material, does not lead to any disruptions in normal reparation processes nor in morpho-functional relations in bone tissue during the remodeling phase, even in extreme experimental conditions of direct contact with damaged bone tissue.

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Хистолошка процена одговора коштаног ткива на материјал за оптурацију канала корена зуба на бази калцијум-хидроксида – експериментална студија

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САЖЕТАК

Увод/Циљ Успех ендодонтског третмана зависи од ефикасног уклањања каналног садржаја, елиминације инфекције и херметичког затварања каналног система материјалом одговарајуће компатибилности.

Циљ овог рада је био да се испита ткивни одговор на коштану имплантацију ендодонтског материјала на бази калцијум-хидроксида у артифицијелно препарисан дефект на мандибули пацова.

Методе Истраживање је спроведено на 40 пацова соја вистар. У пределу између медијалне линије и форамена ментале са леве стране мандибуле препарисан је артифицијелни дефект. Препарисан дефект је остављен да спонтано зараста код животиња контролне групе, док је животињама експерименталне групе у дефект имплантиран материјал за оптурацију канала корена зуба – *Apexit* (*Vivadent*, Лихтенштајн). Ткивни узорци који су се састојали од експерименталног подручја и околне кости анализирани су светлосним микроскопом.

Резултати У почетној фази, 15 дана по апликацији уочени су знаци хроничног запаљења као и проширење Фолкманових и Хаверсових канала. Тридесетог дана од имплантације запажена је остеосинтетска активност. Промене су запажене и на цементним линијама у ширем региону од експерименталног дефекта. После 60 дана од имплантације кост је постепено ремоделисана. Деведесет дана од имплантације је запажен *restitutio ad integrum*.

Закључак *Apexit* не доводи до нарушавања нормалних репарацијских процеса, као и морфофункционалних односа у коштаном ткиву.

Кључне речи: материјали за оптурацију; калцијум-хидроксид; кост; зарастање